



Original Article

Remodelling of retinal on- and off-bipolar cells following experimental retinal detachment

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ABSTRACT

Background: To study the response of ON and OFF bipolar cells in experimental retinal detachment.

Methods: Domestic cat retinas were detached for 7 days. The retinas were prepared for immunocytochemical staining with antibodies to Go alpha (α), glutamate transporter GLT-1, protein kinase C and rod opsin, which serve as markers for ON bipolar cells, OFF bipolar cells, rod bipolar cells and rod photoreceptors, respectively. Both sections and whole-mounts were labelled with antibodies to Go α and GLT-1.

Results: Following 7 days of detachment, ON bipolar cell processes extended into the outer nuclear layer and had neurites extending beyond their target layer into the inner plexiform layer. In contrast, OFF bipolar cell processes were reduced in the outer plexiform layer following detachment.

Conclusion: ON and OFF bipolar cells undergo significant remodelling of their processes in response to retinal detachment, and the ON and OFF pathways may be differentially affected. The remodelling may be due to morphological changes that have previously been shown to occur in photoreceptor synaptic terminals or as a result of loss of synaptic connections due to photoreceptor cell death.

Key words: bipolar cells, remodeling, retinal detachment.

INTRODUCTION

Retinal detachment (RD) induces numerous cellular events in the neural retina, including rapid retraction of rod synaptic terminals, extension of rod bipolar cell and horizontal cell processes, and sprouting of neurites from ganglion cells.^{1–3} It remains unknown whether these responses of second- and third-order neurons include functional changes, but they occur soon after detachment, perhaps in response to a withdrawal of their presynaptic targets: the rod photoreceptor synaptic terminals.^{1–3} Furthermore, a recent study in an animal model of retinitis pigmentosa showed that ectopic synapses form between cone photoreceptor terminals and rod bipolar cell dendrites.⁴ Thus, the second-order neurons may respond to degenerative changes in photoreceptor cells as part of an overall attempt to maintain neuronal connections.

In the current study, we show that ON bipolar cells extend their axonal endings into the ganglion cell layer (GCL) and their dendrites into the outer nuclear layer (ONL) during the period of RD. We also provide evidence that OFF bipolar cells may have a reduced number of processes in the outer plexiform layer (OPL) following detachment.

METHODS

Adult cats (>6 months of age) were used in the study. All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The cats were

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anesthetized with a mixture (1:1) of ketamine hydrochloride (10 mg/kg) (Wako Pure Chemicals Industries, Osaka, Japan) and xylazine hydrochloride (4 mg/kg) (Wako). RD was created in the right eye, as described previously.¹⁻³ Briefly, after lensectomy and vitrectomy, a solution of 0.25% sodium hyaluronate (Healon; Pharmacia, Piscataway, NJ, USA) in balanced salt solution (Alcon, Fort Worth, TX, USA) was infused between the neural retina and the retinal pigment epithelium using a glass micropipette.

Three animals were sacrificed at 7 days after detachment. The enucleated eyes were immersion fixed as eyecups for 10 min in 4% paraformaldehyde in sodium cacodylate buffer (0.1N; pH 7.4) (Wako). Small areas of retina were excised and embedded in low-melting-point agarose (Sigma-Aldrich, St. Louis, MO, USA) for immunocytochemical analysis by confocal microscopy (Bio-Rad 1024; Hercules, CA, USA). The embedded tissue was cut on a Vibratome (Technical Products International, Polysciences; Warrington, PA, USA) and blocked overnight in normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; 1:20) at 4°C. Sections were then incubated with primary antibodies overnight at 4°C on a rotator. The primary antibodies used in this study were a mouse monoclonal antibody to $G\alpha$ (Chemicon, Temecula, CA; 1:400), a rabbit polyclonal antibody to GLT-1 (Chemicon; 1:100), a mouse monoclonal antibody to rhodopsin (Chemicon; 1:400) and a rabbit polyclonal antibody to protein kinase C (PKC) (Chemicon; 1:400). All antibody solutions were made in PBTA: 0.1 M phosphate-buffered saline containing 0.5% bovine serum albumin (Fisher Scientific, Pittsburgh, PA, USA), 0.1% Triton X-100 (Boehringer-Mannheim, Indianapolis, IN, USA) and 0.1% sodium azide (Sigma). Sections were rinsed in PBTA and then incubated with donkey anti-mouse IgG conjugated to the fluorochrome Cy3 ($G\alpha$, GLT-1) and donkey

anti-rabbit IgG conjugated to the fluorochrome Cy2 (PKC) (Jackson ImmunoResearch Laboratories) overnight at 4°C on a rotator. The sections were mounted in mounting medium for fluorescence (Vectashield, Vector Laboratories, Inc., Burlingame, CA, USA) and viewed on a laser scanning confocal microscope.

Retinal whole-mount immunocytochemistry was performed using the following protocol. Retinas were removed and rinsed three times in phosphate-buffered saline before incubation in blocking solution for 6 h and then with anti- $G\alpha$ or anti-GLT-1 (both at 1:500) for 2 days. After incubation, the sections were washed in PBTA and then incubated with anti-mouse or anti-rabbit IgG conjugated to Cy3 for 24 h. The retinal whole-mounts were rinsed in PBTA and mounted with the photoreceptor side up.

RESULTS

Immunolabelling with anti- $G\alpha$ in normal retinas was observed in the inner plexiform layer (IPL) and OPL and in dendrites and cell bodies of a subset of bipolar cells (Fig. 1a). Immunocytochemical labelling of normal retinas with anti-PKC occurred in rod bipolar cells (Fig. 1b). Robust labelling was present in the rod bipolar cell bodies, axons and axon terminals. In double labelling of normal retinas with anti- $G\alpha$ and anti-PKC, two populations of bipolar cells were apparent. The population labelled with both antibodies was assumed to be rod bipolar cells because anti-PKC is specific to these cells. The second population labelled only with $G\alpha$ antibody was assumed to be ON-cone bipolar cells (Fig. 1c; red). These findings are consistent with previous results, thus indicating that the staining pattern identifies the ON bipolar cells.⁵

Immunostaining with anti-GLT-1 occurred in the IPL, OPL, some bipolar cells and cone photoreceptor cells (Fig. 2a). Previous results showed that the

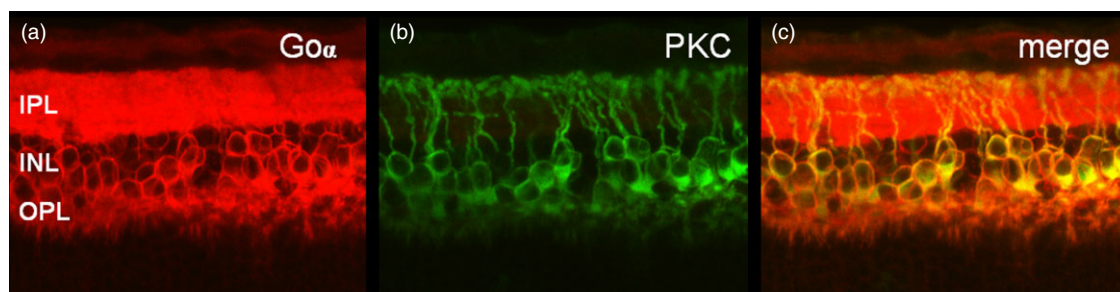


Figure 1. Anti- $G\alpha$ and protein kinase C (PKC) labelling of normal retinal sections. Anti- $G\alpha$ labelling occurs in the inner and outer plexiform layer (IPL and OPL) and in cell bodies in the inner nuclear layer (INL) (a). Anti-PKC labelling is present in the cell bodies of rod bipolar cells in the INL and their dendrites in the OPL (b). A merged image shows that some $G\alpha$ -positive cells are also labelled with anti-PKC and therefore appear yellow, whereas ON-cone bipolar cells appear only red (c). IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar, 20 μ m.

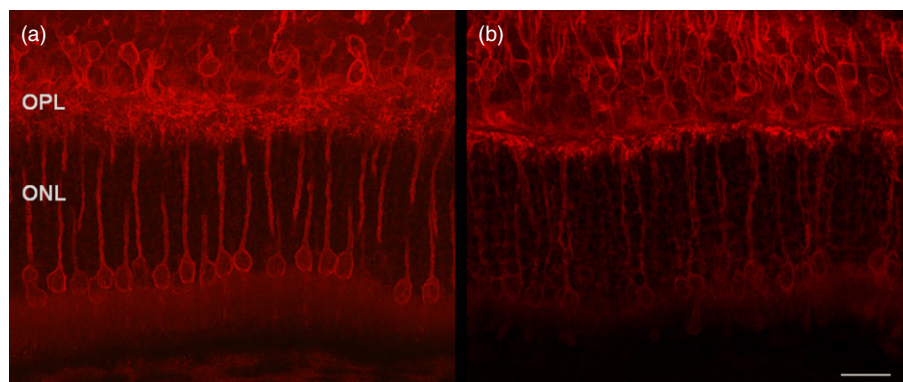


Figure 2. Anti-GLT-1 labelling of normal (a) and detached (b) retinal sections. Anti-GLT-1-labelled bipolar dendrites appear to show faint labelling in the outer plexiform layer (OPL) after detachment (b), in comparison to a normal retina (a). Faint labelling is also present in the cone axon and cell bodies (b). OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar, 20 μ m.

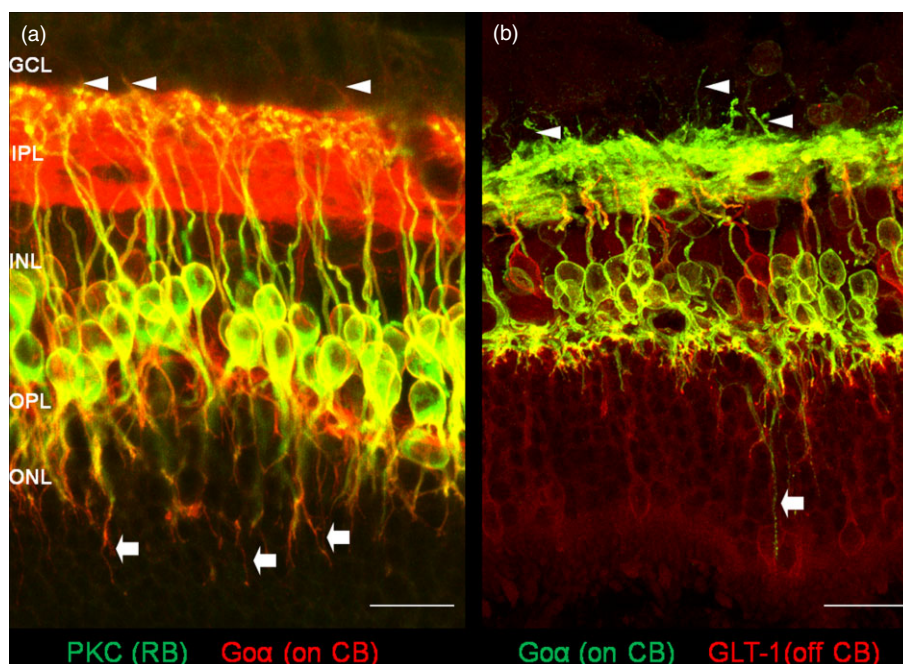


Figure 3. Anti-Go α , protein kinase C (PKC), and GLT-1 labelling of detached retinal sections. Anti-Go α -labelled bipolar dendrites (a: red; b: green, arrows) extend beyond the normal boundaries of the outer plexiform layer (OPL) and into the outer nuclear layer (ONL) (a, b). Anti-PKC-labelled bipolar dendrites (yellow) also extend into the ONL (a). Anti-Go α labelled neurites from bipolar cell axon terminals also extend beyond the inner plexiform layer (IPL) into the ganglion cell layer (GCL) (arrowheads). Anti-GLT-1 labelling (red) was observed in bipolar cell bodies, axons, cone cell bodies, and cone axons (b). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar, 20 μ m.

labelled bipolar cells are OFF bipolar cells.⁶ Thus, the results in the feline retina are consistent with reports indicating that anti-GLT-1 specifically labels a subclass of bipolar cells identified as OFF bipolar cells.⁶ In detached retinas, there were no GLT-1-labelled OFF bipolar cell (red) neurite extensions into the ONL (Fig. 2b). There also seemed to be an overall decrease in intensity of labelling with the GLT-1 antibody after detachment (Fig. 2b; compare with Fig. 2a).

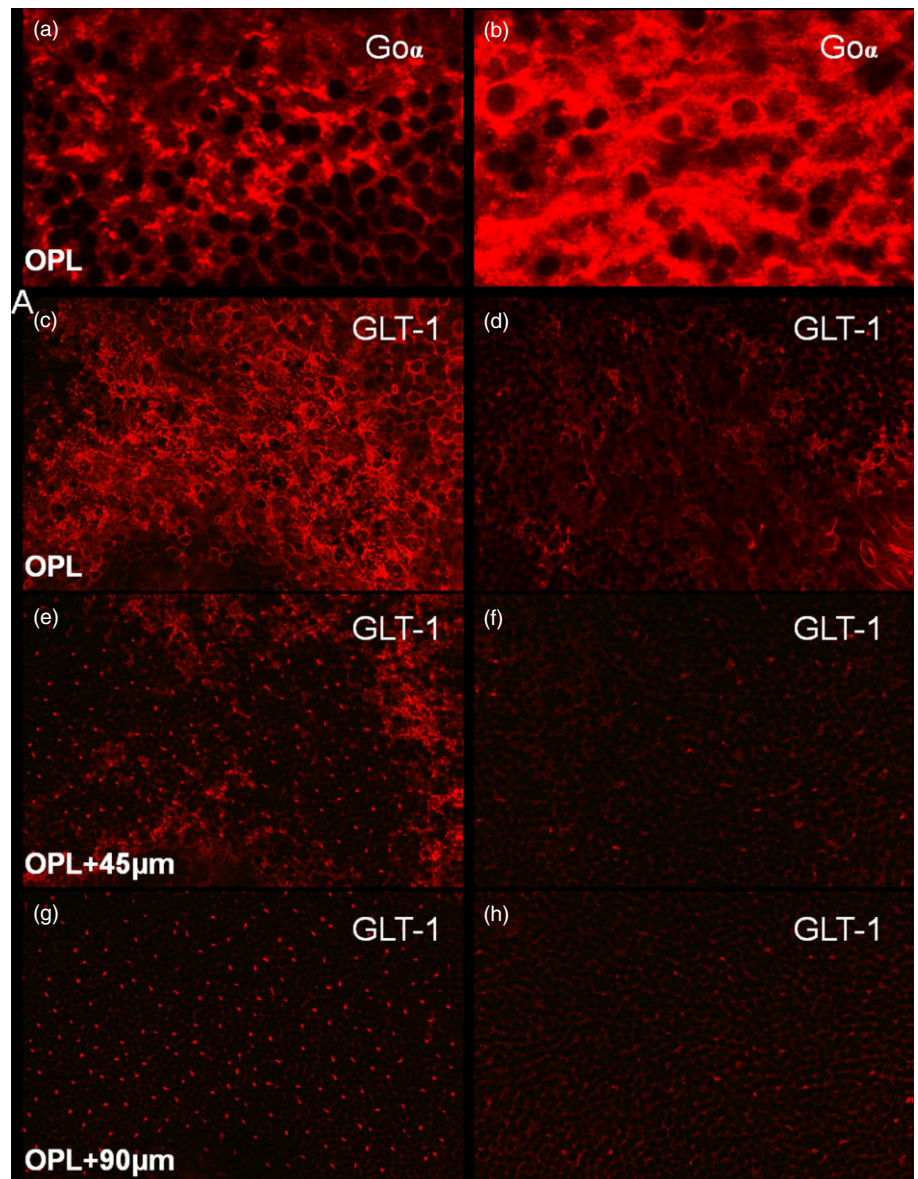
At 7 days after detachment, staining showed both double-labelled rod bipolar cell (yellow) and ON-cone bipolar cell (red) neurite extensions into the ONL (Fig. 3a). One such neurite extends to the level of cone cell bodies on the outer border of the ONL and appears to terminate adjacent to a cone faintly labelled with anti-GLT-1 (Fig. 3b). ON-cone bipolar cell axons may also sprout neurites because we observed appropriately labelled processes

extending out of the heavily labelled IPL and into the GCL (Fig. 3a,b; arrowheads).

To better determine the response of the anti-Go α - and GLT-1-labelled dendrites in the OPL and ONL, whole-mount immunocytochemistry was performed using anti-Go α or anti-GLT-1 (Fig. 4a–h). In the detached retina, labelling with the Go α antibody became more prominent in both the outer part of the OPL and the inner part of the ONL, in comparison with the normal retina (Fig. 4a,b). In contrast, labelling with the GLT-1 antibody was greatly reduced in the OPL, showing a patchiness that differed greatly from the homogenous labelling in the non-detached retina (Fig. 4d; compare with Fig. 4c). The labelling intensity in the ONL also decreased in the detached retina (Fig. 4e–h).

Double-labelling experiments with anti-GLT-1 and anti-rhodopsin somewhat surprisingly showed significant areas of overlap between the two

Figure 4. Normal (a, c, e, g) and detached (b, d, f, h) retinal whole-mounts labelled with anti-Go α (a, b) and anti-GLT-1 (c–h). Anti-GLT-1-labelled structures in the outer nuclear layer (ONL) were sampled at 45 μ m (e, f) and 90 μ m (g, h) distal to the outer plexiform layer (OPL). In detached retina, anti-Go α labelling (b) was increased in the OPL compared with (a), but anti-GLT-1 labelling (d) was decreased in the OPL compared with (c). In the ONL, labelling was faint after detachment (f, h) compared with a normal retina (e, g). OPL, outer plexiform layer.



antibodies in the layer of rod terminals (Fig. 5b). In the detached retina, rod opsin redistributes to the plasma membrane such that it eventually outlines the entire rod cell (Fig. 5a,b). At higher magnification, it appeared that in some cases, yellow labelling was created by red OFF bipolar cell dendrites terminating adjacent to green rod synaptic terminals.

DISCUSSION

This study on RD using specific markers for ON and OFF bipolar cells confirmed a previous finding that neurite growth into the ONL occurs from the ON-cone, as well as from rod bipolar cells.¹ At the same time, the ON-cone bipolar cell axons appear to grow neurites into the GCL, a novel event that has not been identified previously in rod bipolar cells.

Furthermore, we newly found that OFF bipolar cells may retract dendrites in response to detachment, as indicated by a decrease in GLT-1 labelling. A similar observation has been made in the inherited retinal degeneration rd/rd mouse, but with the conclusion that bipolar cell dendrites retracted as degeneration proceeded.^{7,8} Thus, the present findings indicate significant changes in the ON and OFF bipolar cells in response to RD. However, neurite sprouting may be a common theme in many cell types because deafferentation by degeneration or detachment may induce remodelling through removal of glutamate (GLU) and Ca²⁺ coordinated signalling.⁹

Anti-Go α labels both rod bipolar cells and ON-cone bipolar cells,⁵ but we were able to observe ON-cone bipolar cells exclusively by double-labelling with anti-Go α and anti-PKC. This is

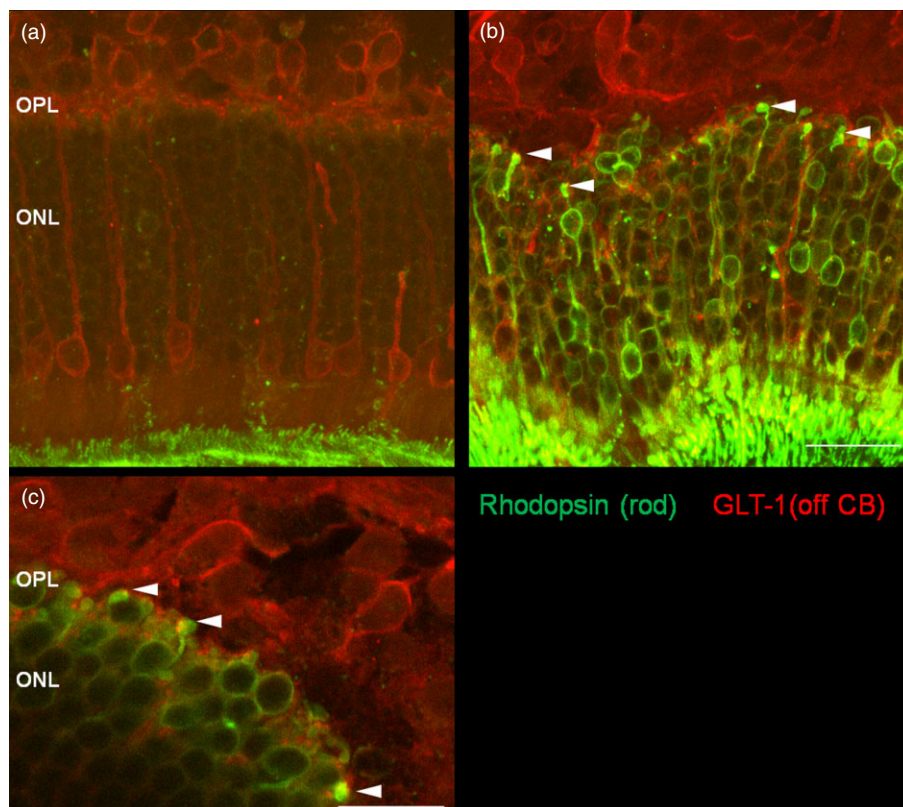


Figure 5. Double-label immunocytochemical analysis using anti-GLT-1 (red) and anti-rhodopsin (green) in normal (a) and detached (b, c) retinal sections. A higher magnification of the outer plexiform layer (OPL) in a detached retina is shown in (c). Some rod terminals had associated bright yellow fluorescent 'spots' (arrowheads), indicating colocalization of GLT-1 and rhodopsin in these subcellular regions in the detached retina (b, c). OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar, 20 μ m.

because anti-PKC labels only rod bipolar cells, and thus double-labelling identifies the population of rod bipolar cells. Double-labelled processes were observed in the ONL and were presumably a result of growth of both the rod bipolar dendrites and axon terminals. Neurite outgrowth into the ONL also occurs from both rod bipolar cells and ON-cone bipolar cells, but neurite outgrowth into the GCL may occur only in the cone pathway, perhaps indicating that the rod- and cone-driven pathways are differentially affected by RD. If neuronal remodelling has an effect on visual recovery, the neuronal repair mechanisms may also differ for the two pathways.

The OFF bipolar cell response to RD was studied using anti-GLT-1. This antibody is known to label OFF bipolar cells that correspond morphologically to type cb1 cone bipolar cells, which are a major source of input to OFF-beta ganglion cells in the cat retina.⁶ Interestingly, the labelling intensity in the OPL was greatly reduced after detachment, in contrast with anti-Go α staining, suggesting that the ON and OFF pathways may be differentially affected in RD.

RD induces rapid and specific changes in the distribution of GLU.¹⁰ GLU-immunoreactivity (IR) in cones has been found to be significantly decreased in detached retinal regions at all time points,¹¹ with the conclusion that depolarization-induced reversal of excitatory amino acid transporters may be a possible

mechanism for GLU depletion. It is unclear if the marked decrease in GLT-1 IR that we observed in cones is associated with the reduced GLU-IR. Determining the exact mechanism through which cones undergo GLU depletion following RD will require further studies.

In photoreceptor degeneration induced by mutated β PDE or rhodopsin in swine, Peng *et al.* demonstrated that rod bipolar cells receive synaptic input from cone photoreceptors.⁴ This also occasionally occurs in normal mammalian retina.¹² A small percentage of OFF bipolar cells in the rodent and feline retina also make synaptic contact with rods.^{6,13} In the current study, we observed a close apposition of OFF bipolar cell dendrites to rod synaptic terminals in the degenerating feline retina. Thus, our immunocytochemistry provides confirmatory evidence that OFF bipolar dendrites are making contact with rod photoreceptors in the detached feline retina. Interestingly, the synaptic contact between OFF bipolar cells and rods appears to be silent in response to detachment. The functional significance of this contact in RD is unknown, but it may be implicated in pooling of rod signals.

In conclusion, the results of this study add to the growing evidence for remodelling of second-order neurons under conditions that result in photoreceptor degeneration, including RD. Neural plasticity from ON and OFF bipolar cells may be of significance for

recovery of vision after retinal reattachment and other forms of photoreceptor degeneration.

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